Method of producing *thy* A⁻ strains of *Vibrio cholerae*, such strains and their use.

The present invention relates to a method of producing *thy* A⁻ strains of *Vibrio cholerae,* such strains and their use. The invention particularly relates to a strain of *Vibrio cholerae* that has been deprived of its *thy* A gene in the chromosome, i.e. a Δ *thy* A strain lacking the functionality of the *thy* A gene. This strain may comprise one or several episomal autonomously replicating DNA elements, such as plasmids, having an optionally foreign, e.g. *E. coli*, functional *thy* A gene that enables the strain to grow in the absence of thymine in the growth medium, and optionally having a structural gene encoding a homologous or heterologous protein. The invention further relates to *thy* A nucleotide sequences and proteins encoded by them, and a vaccine comprising as an immunizing component a *Vibrio cholerae* Δ *thy* A strain of the invention or a *thy* A⁻ strain of *V. cholerae* produced by the method of the invention.

Background.

The expression of recombinant genes in bacterial hosts is most often achieved by the introduction of episomal self-replicating elements (*e.g.* plasmids) that encode the structural gene of the protein of interest under the control of an appropriate promoter, into host bacteria. Such plasmids are most commonly maintained by the inclusion of selective marker genes that encode proteins that confer resistance to specific antibiotics (such as ampicillin, chloramphenicol, kanamycin, tetracycline etc.). They are then maintained in the host by addition of the appropriate antibiotic to the culture medium.

Stable maintenance of plasmids in host strains often requires the addition of the appropriate antibiotic selection without which they may segregate out giving rise to significant numbers of cells in any culture, that are devoid of plasmid and therefore cannot express the desired product.

However, the use of antibiotics in the production of recombinant proteins is undesirable for a number of reasons. Apart from the obvious increase in costs arising from the need to add them as a supplement to the growth medium, the use of antibiotics is considered a problem in the production of any recombinant protein intended for human or veterinary use. This is primarily for three reasons. Firstly, residual antibiotics can, in sensitive individuals, cause severe allergic reactions. Secondly, there is the possibility of selection for antibiotic resistant bacteria in the natural bacterial flora of those using the product, and finally, DNA encoding the antibiotic resistance may also be transferred to

sensitive bacteria in individuals using the product, thereby also spreading undesired antibiotic resistance in a cohort.

There are already inventions dealing with this problem, one such is the *par* gene which will effectively kill all cells that do not retain a copy of the plasmid after each cell division [1].

Another patent application [2], which touches on the invention described herein, was based on the knowledge of the *thy*A DNA sequence in *E. coli*. The authors introduced the *thy*A gene on a plasmid but used host strains that were spontaneous *thy*A$^-$ mutants selected on the bases of trimethoprim resistance. Such mutants are not well defined (carrying point mutations or small deletions) and may revert to the wild-type (*i.e.* *thy*A$^+$) at unacceptably high frequencies. This would lead to that the host bacteria could eliminate the plasmid and hence lose, or not give consistent and reliable, production of the desired recombinant product. An additional problem with trimethoprim selection is the possibility that resulting thymine dependence may arise due to a mutation in the dihydrofolate reductase (*fol*A) gene and hence not be complemented by a plasmid-borne *thy*A gene [3]. This patent application has been discontinued at least in Europe.

The use of *V.cholerae* for expression of recombinant genes has been shown to be advantageous over other prokaryotic expression systems in common use in that specific recombinant products may be produced in large quantities and secreted into the culture medium, thereby facilitating downstream purification procedures. This is in contrast to *E.coli* where the product often assembles in the periplasmic space [4]. One important factor endowing *V.cholerae* with this property is the *eps* genes in *V.cholerae* [5].

Thymidylate synthetase encoded by the *thy*A gene of *Escherichia coli* and other bacteria catalyses the methylation of deoxyuridylate (dUMP) to deoxythymidylate (dTMP) and is an essential enzyme in the biosynthesis of deoxyribothymidine triphosphate (dTTP) for incorporation into DNA. In the absence of this enzyme the bacteria become dependent upon an external source of thymine which is incorporated into dTTP by a salvage pathway encoded by the *deo* genes [6].

Spontaneous mutants that are *thy*A$^-$ can be readily isolated on the basis of trimethoprim resistance. This antibiotic inhibits tetrahydrofolate regeneration from dihydrofolate produced by thymidylate synthetase-catalysed dTMP synthesis. Thus, if the cells are *thy*A$^-$ they become thymine dependent but no longer deplete the tetrahydrofolate pool in the presence of trimethoprim.

Description of the invention

The present invention is, in its different aspects, based on the novel nucleotide sequence of the *thy*A gene in *Vibrio cholerae*. A useful application of the *thy*A gene is e.g. in maintenance of recombinant plasmids employed in the overproduction of recombinant proteins in *V. cholerae*, and in the use of the sequence for insertion of foreign genes in a selectable and site-specific manner into the *V. cholerae* chromosome .

One aspect of the invention is directed to a method of producing a *thy* A$^-$ strain of *Vibrio cholerae* comprising the step of site-directed mutagenesis in the *V. cholerae* chromosome for the deletion and/or insertion of gene nucleotides at the locus of the *thy* A gene having essentially the nucleotide sequence SEQ ID NO: 1 of FIG. 1.

The expression "having essentially the nucleotide sequence" in this specification and claims is intended to comprise nucleotide sequences which have some natural or unnatural nucleotide extensions, truncations, deletions or additions that do not interfere with the natural function of the nucleotide sequence in question.

Another aspect of the invention is directed to a *Vibrio cholerae thy* A$^-$ strain which is a Δ *thy* A strain lacking the functionality of the *thy* A gene.

In an embodiment of this aspect of the invention the Δ *thy* A strain of *V. cholerae* comprises one or several episomal autonomously replicating DNA elements having a functional *thy* A gene that enables the strain to grow in the absence of thymine in the growth medium.

In a preferred embodiment the episomal autonomously replicating DNA element is a plasmid.

In another preferred embodiment the Δ *thy* A strain according to the invention comprises in an episomal autonomously replicating DNA element, especially a plasmid, a foreign *thy* A gene, such as an *E. coli* gene.

In a particularly preferred embodiment of this aspect of the invention the Δ *thy* A strain according to the invention comprises in one or several episomal autonomously replicating DNA elements, especially plasmids, in addition to a foreign *thy* A gene, such as an *E. coli* gene, also a structural gene encoding a homologous or heterologous protein, such as heat labile enterotoxin B-subunit of *Escherichia coli* (LTB) or *Schistosoma japonicum* glutathione S-transferase 26 kD protein (GST 26 kD).

A third aspect of the invention is directed to a nucleotide sequence of a 5′-flanking region of a structural *thy* A gene of *Vibrio cholerae* having essentially the nucleotide sequence SEQ ID NO: 2 of FIG. 2.

A fourth aspect of the invention is directed to a nucleotide sequence of a 3′-flanking region of a structural *thy* A gene of *Vibrio cholerae* having essentially the nucleotide sequence SEQ ID NO: 3 of FIG. 3.

The nucleotide sequence SEQ ID NO: 1, is useful for insertion of foreign genes in a selectable and site-specific manner into the *V. cholerae* chromosome, and for site-directed mutagenesis in the production of *Vibrio cholerae thy* A⁻ strains.

A fifth aspect of the invention is directed to a protein encoded by a nucleotide sequence of a *thy* A gene of *Vibrio cholerae* according to the invention, such as a protein having the amino-acid sequence SEQ ID NO: 4 of FIG. 4.

A sixth aspect of the invention is directed to a protein encoded by a nucleotide sequence of a 5′- flanking region of a structural *thy* A gene of *Vibrio cholerae* according to the invention, such as the protein having the amino-acid sequence SEQ ID NO: 5 of FIG. 5.

The proteins according to the fifth and sixth aspect of the invention are each useful for research purposes, and potential targets for anti-microbial therapy.

A seventh aspect of the invention is directed to a vaccine comprising as an immunising component a *Vibrio cholerae* Δ *thy* A strain according to the invention or a *thy* A⁻ strain of *Vibrio cholerae* produced by the method of the invention. The vaccine will be used for prophylactic and therapeutic treatment of cholera and optionally other infectious diseases, especially in cases where the used strain has been engineered to express foreign proteins. The vaccine will in addition to the immunising component(s) comprise a vehicle, such as physiological saline solution, and other components frequently used in vaccines such as buffers and adjuvants. Useful vehicles, buffers, adjuvants and other components are disclosed in e.g. the European and US Pharmacopoeia.

Short description of the drawings

Figure 1 shows the nucleotide sequence SEQ ID NO:1 of the *thy* A gene of *Vibrio cholerae*.

Figure 2 shows the nucleotide sequence SEQ ID NO:2 of the 5′-flanking region of the structural *thy* A gene of *Vibrio cholerae*.

Figure 3 shows the nucleotide sequence SEQ ID NO:3 of the 3′-flanking region of the structural *thy* A gene of *Vibrio cholerae*.

Figure 4 shows the amino-acid sequence SEQ ID NO:4 of the protein encoded by the structural *thy* A gene of *Vibrio cholerae* .

Figure 5 shows the amino-acid sequence SEQ ID NO:5 of the protein encoded by the 5´-flanking region of the structural *thy* A gene of *Vibrio cholerae*.

Figure 6 shows the cloning of a *Eco*RI/*Hind*III fragment containing the *V.cholerae thy*A gene in pUC19.

Figure 7 shows a comparison of *thy*A gene products from *E. coli* [16], *V. cholerae* and *H. influenzae* [17] showing the high degree of homology between *V. cholerae* and *H. influenzae* compared with *E. coli*.

Figure 8 shows the insertion of a Kan^R-resistance gene block in the *Pst*I site of the *V.cholerae thy*A gene in pUC19.

Figure 9 shows PCR to generate a *thy*A -Kan fragment with *Xba*I ends.

Figure 10 shows ligation of the *thy*A-Kan fragment with *Xba*I ends in plasmid pNQ705.

Figure 11 shows partial deletion of the *thy*A gene and the start of the Kan gene in pNEB193.

Figure 12 shows *Xba*I cleavage to excise the Δ*thy*A Δkan gene from pNEB193, ligation into *Xba*I restricted pDM4.

Figure 13 shows an outline of a strategy to completely delete the *thy*A gene of *V. cholerae.*

Figure 14 shows insertion of the 5' region upstream of *thy*A in pMT-SUICIDE 1; generation of pMT with 5 prim.

Figure 15 shows insertion of the 3' region downstream of *thy*A in pMT with 5 prim; generation of pMT Δ*thy*A *V.cholerae.*

Figure 16 shows the expression vector pMT-eltB(thyA) used for expression of LTB in *V. cholerae* JS1569 Δ*thyA.*

Figure 17 shows the expression vector pMT-GST(thyA) used for expression of GST in *V. cholerae* JS1569 Δ*thy*A.

Description of experiments

Strategy employed

In order to produce defined *thy*A mutants of *V. cholerae* that could be used as suitable production strains for recombinant proteins encoded on plasmids maintained by *thy*A complementation, it was first necessary to clone and characterise the wild-type gene and its

5' and 3' flanking regions. Our strategy was to first clone the *thy*A gene of *V. cholerae* on a plasmid, on the basis of complementation of the *thy*A auxotrophy in a strain of *E.coli* K12. Restriction analysis and subcloning experiments were done in order to locate the *thy*A structural gene on the large DNA fragment initially obtained. The appropriate region containing the *thy*A gene and it's 5' and 3' flanking regions gene was then sequenced.

To verify that one of the sequenced genes was in fact the *thy*A gene of *V. cholerae*, homology comparisons were made with *thy*A sequences from other organisms. The cloned gene could also complement the *thy*A phenotype of a *V. cholerae* mutant strain that had been selected on the basis of trimethoprim resistance. Sequence analysis of this mutant showed that it did indeed have a single base change in the gene we had identified as *thy*A, which resulted in a stop codon giving a non-functional truncated gene product.

Knowledge of the *thy*A sequence and that of the region surrounding it allowed the use of suitable suicide vectors for site-directed mutagenesis. Strategies considered were (a) insertional inactivation (b) a combination of insertional inactivation and gene deletion and (c) removal of the entire gene:

(a) Insertional inactivation of the *thy*A gene was achieved by insertion of a Kan^R gene block (with the suicide vector pNQ705 [14].

(b) A deletion of approximately 400 bp was made in the strain carrying the Kan^R geneblock that removed 200 bp each from the *thy*A gene upstream of the insertion site and from the kanamycin resistance gene which was thereby inactivated. We thus obtained a deleted *thy*A gene where the deletion was in the central part of the gene and followed by an insertion of a non-coding region of DNA. This construct was inserted into the *V .cholerae* chromosome using the suicide vector pDM4 and resulted in a strain called JS1569 Δ*thy*AΔKan.

(c) Complete removal of the *thy*A gene was done by ligating together the regions flanking the structural gene, taking care not to disrupt other open reading-frames (disruption of the adjacent *lgt* gene is also lethal). The DNA carrying the deletion was cloned into a novel suicide vector (PMT-SUICIDE-1) used for insertion of the sequence into the *V. cholerae* chromosome. The resulting strain is called JS1569 Δ*thy*A.

For expression of recombinant genes in these Δ*thy*A strains of *V. cholerae*, two expression vectors were constructed. Each consisted of the *thy*A gene from *E. coli*, the origin of replication of the general purpose high copy-number vector pUC19, the *tac* promotor and the rho-independent *trp*A transcription terminator. In one of the two vectors the $lacI^q$ gene

had been inserted in order to regulate expression from the *tac* promotor which also contained the *lac* operator sequence.

Two genes were cloned into these plasmids and expressed in the newly generated *thy*A -deleted strain of *V. cholerae*; JS1569Δ*thy*A. The first encoded the B subunit of human heat-labile enterotoxin from *E. coli* (LTB) (Figure 16), the second was the sj26 glutathione-S-transferase (GST) from *Schistosoma japonicum* (Figure 17).

LTB is similar in structure to the B subunit of cholera toxin naturally produced by the host strain and was secreted into the growth medium. The other protein is eukaryotic in origin, coming from the Asian Liver Fluke. Sj26 GST is known to express to high levels in *E. coli* and accumulates in the cytoplasm. Expression of the two recombinant proteins was assessed on the basis of GM1 ELISA of the culture supernatant in the case of LTB and a commercially available assay in the case of GST. Both proteins were also analysed on the basis of SDS-PAGE and Western blots.

Origin of the *thy*A gene

The *thy*A gene was cloned from strain ***V.cholerae*** JS1569. This strain originates from the *V.cholerae* Inaba strain 569B of the classical biotype (ATCC No 25870). The strain has a deletion in the *ctx*A gene [7] and has been made rifampicin resistant [8].

Cloning of a 1.4 kB *Hind*III/*Eco*RI fragment encompassing the *V.cholerae thy*A gene.

Chromosomal DNA prepared by the CTAB method [9] was digested to completion with the restricion enzyme *Hind*III.

The digested DNA was ligated into the general purpose vector plasmid pBR322 (New England Biolabs Inc. Beverly, MA USA) which had been digested with *Hind*III and treated with alkaline phosphatase.

The ligation mixture was electroporated [10] into a *E.coli* HB101 strain that was phenotypically ThyA$^-$ (selected on the basis of trimethoprim resistance) and the culture spread onto modifed Syncase (MS) agar plates [11] supplemented with 50 μg/ml ampicillin, but containing no thymine. Thus transformants were selected both on the basis of plasmid acquisition and the presence of a functional *thy*A gene.

Colonies that grew up were streaked out to single colonies on the same type of agar plates, and then grown up in MS broth supplemented with ampicillin. Plasmid DNA was prepared by "Wizard miniprepps" (ProMega Corp. Madison Wis.) and digested with *Hind*III. A fragment of approx. 10-12 kB was isolated, this clone was namned ThyA B2.

To reduce the size of the fragment, the plasmid was cut with *Eco*RI and religated using T4 ligase. The ligated DNA was again electroporated into the *E.coli* strain described above using the same selective conditions for growth of transformants.

Colonies resulting from this experiment were isolated as described above and plasmid DNA purified and anlysed by double digest with *Eco*RI and *Hind*III. A DNA fragment of approximately 1.4 kb remained which retained the ability to complement the *thy*A mutation in the *E. coli* host strain. This fragment was cloned into the plasmid pUC19 (New England Biolabs) that had been digested with the same two enzymes and treated with alkaline phosphatase. Following electroporation, transformants from the experiment were isolated and characterised as described above. This clone was called ThyA 1:2 (Figure 6).

Verification that the 1.4 kB *Hind*III/*Eco*RI fragment contains the *thy*A gene.

Southern blot analysis. To verify that the cloned fragment was indeed from *V.cholerae* chromosomal origin, DNA from strain JS1569 was digested to completion with *Hind*III and *Eco*RI and *Hind*III. The DNA fragements were resolved by agarose electrophoresis together with *Hind*III digested clone ThyA B2 and *Eco*RI and *Hind*III digested clone ThyA 1:2.

After electrophoresis the DNA was transferred to a Nylonmembrane, immobilised by UV irradiation and hybridised (under stringent conditions) with the 1.5 kB fragment excised from clone ThyA 1:2 that had been labelled with ^{32}P dCTP using Amershams Multiprime kit.

Results. In both *Hind*III digested chromosomal DNA and in *Hind*III digested clone ThyA B2 an approx. 10 kB band was evident. Likewise in *Eco*RI/*Hind*III digested chromosomal DNA and clone ThyA 1:2 plasmid DNA a 1.4 kB band was evident (data not shown). These data demonstrated that the cloned fragment was derived from *V.cholerae* JS1569 DNA.

Transformation of JS1569 ThyA$^-$ with the plasmid ThyA 1:2.

To verify that the 1.4 B cloned *Eco*RI/*Hind*III fragment could support growth of phentotypically ThyA$^-$ *V.cholerae*, a thymine dependent mutant of JS1569 (*V.cholerae* JS1569 4.4) was electroporated with the plasmid ThyA 1:2. Electroporation and selective media were as described above. JS1569 4.4 does not grow on MS medium without the addition of thymine.

Results. Colonies of JS1569 4.4 were isolated that grew in the absence of thymine. All were shown to harbour the ThyA 1:2 plasmid, thus supporting the assumption that the cloned fragment contained the *thy*A gene from *V.cholerae*.

DNA sequencing of the plasmid ThyA 1:2. Plasmid DNA was sequenced by the dideoxy chain termination method [12] using the ABI PRISM™ Dye terminator cycle sequencing kit (Perkin Elmer). Both commercially available as well as custom made primers were used. The DNA sequences were analysed on an ABI PRISM 373 automatic sequencer (Perkin Elmer). Data were analysed using the AutoAssembler Software package (Perkin Elmer). Homology searches with the found DNA sequence were done with the GCG program [13].

Results. The best homologies were with thymidylate synthetases from various species. Note that the homology with *E.coli* thymidylate synthetase is rather weak. (Figure 7)

Strategy for deletion of the *thy*A gene in *V.cholerae* JS1569.

Two different strategies were used for obtaining defined *thy*A mutants of *V. cholerae* JS1569, the first involved inactivation of the *thy*A gene by insertion of a Kan^R gene block followed by partial deletion of the *thy*A gene and the Kan^R gene block.

The second strategy was directed to completely delete the *thy*A gene from the chromosome by means of a novel suicide vector pMT SUICIDE-1. This vector contains the 5' and 3' flanking regions of the *thy*A gene as well as the R6K origin of replication and the RP4 *mob* genes.

To replace the *thy*A gene of strain JS1569 we decided to use the already thymine-dependent JS1569 4.4 since preliminary experiments indicated that there is a strong selective disadvantage to go from wildtype to thymine dependence even in the presence of high levels of exogeneous thymine.

Inactivation of the *thy*A gene by insertion of a Kan^R gene block

Our strategy involved inactivation of the *thy*A gene by insertion of a kanamycin resistance gene into a unique *Pst*I site in the *thy*A gene in the form of a Kan^R gene block (Pharmacia) (Figure 8). This construct was amplified by PCR (Expand™High Fidelity PCR system Boehringer Mannheim) with primers that incorporate *Xba*I ends so that it could be transferred into the suicide plasmid pNQ705 [14] which carries a unique *Xba*I site and the chloramphenicol resistance gene.

The following primers were used for PCR amplification of the insertionally inactiviated gene:

ThyA-10: 5'GCT **CTA GAG** CCT TAG AAG GCG TGG TTC3'
corresponding to bases 557 to 575 in SEQ ID NO: 2 (Figure 2) with an added *Xba*I site (in bold)
and
ThyA-11: 5'GCT **CTA GAG** CTA CGG TCT TGA TTT ACG GTA T3'
corresponding to the complementary sequence of bases 235 to 257 in SEQ ID NO:2 (Figure 3) with an added *Xba*I site (in bold) (Figure 9 + 10).

The resulting plasmid was then transferred to the *E.coli* S-17 that was used in conjugation experiments.

Since the recipient strain JS1569 4.4 is rifampicin resistant and chloramphenicol sensitve and the donor strain *E.coli* S-17 is both chloramphenicol and kanamycin resistant, transconjugants were selected by selection for resistance to both rifampicin and kanamycin.

The resulting *V. cholerae* strains however would also be chloramphenicol resistant since the entire plasmid would initially be inserted into the chromosome.

Exconjugants that had incorporated the inactivated *thy*A gene carrying the Kan^R geneblock into the chromosome and lost the pNQ705 plasmid could then be selected among those that were chloramphenicol sensitive but remained kanamycin resistant.

To verify insertion of the Kanamycin reiststance gene in the *thy*A gene the entire *thy*A gene was PCR amplified with primers thyA-10 and thyA-11, and the size of the resulting fragment compared to that of the native *thy*A gene . The expected *thy*A fragment of 2.6 kb compared to that of the native *thy*A gene of 1.4 kb was found.

Results. Exconjugants were shown to be kanamycin resistant, chloramphenicol sensitive and when amplified by PCR, shown to have incorporated the kanamycin resistance gene block into the chromosome. Sequencing of the amplified fragment showed that the only defect in the gene was due to the insertion of the kanamycin gene. This indicated that the recombination event that had incorporated the insertionally inactivated gene into the chromosome had also eliminated the point mutation that had made the recipient strain (JS1569 4.4) thymine dependent. Growth of the resulting strain was only observed if the growth medium was supplemented with thymine (200 μg/ml).

Partial deletion of the *thy*A gene and the Kan^R gene block

To further ensure a nonreversible *thy*A mutation the insertionally inactivated *thy*A was subcloned as a *Xba*I fragment into pNEB 193 (New England Biolabs). PCR

primers were designed that deleted 209 basepairs from the *thy*A gene and removed 261 basepairs from the Kan^R geneblock.

Thus the *thy*A gene was further disrupted and that the kanamycin resistance gene was also inactivated (by removal of the start of the coding region). The overall result of this procedure was a strain carrying a deleted *thy*A gene that also contained an insertion of noncoding DNA.

ThyA-14: $^{5'}$GGG GG**C TCG AG**G GGC ACA TCA CAT GAA$^{3'}$
ThyA-15: $^{5'}$CCC CC**C TCG AG**C GCC AGA GTT GTT TCT GAA$^{3'}$

Letters in **bold** indicate *Xho*I cleavage sites (Figure 11).

After PCR amplification a DNA fragment was obtained encompassing the entire plasmid with exception of the deleted region. The amplified DNA was digested with *Xho*I, self ligated and transformed into *E.coli* HB101. Colonies were selected for on plates containing ampicillin. Individual colonies were selected and restreaked. Small-scale plasmid preparations from individual colonies yielded the expected restriction patterns when anlysed with *Xba*I, ***Xho*I**, *Hind*III and *Rsa*I restriction enzymes.

The incomplete *thy*A gene carrying an inactivated kanamycin resistance gene was cut out from the vector by *Xba*I digestion, purified and ligated into pDM4 [15] (Figure 12). PDM4 is a suicide vector derived from pNQ705 containing the *Sac*BR gene from *Bacillus subtilis* and a modified multicloning site.

After transfer of the pDM4 (Δ*thy*AΔKan) plasmid to the *E.coli* S-17 strain a transconjugation experiment was performed. This time the *V.cholerae* JS1569 *thy*AKan strain obtained above was used as recipient strain.

The mating was done as described above with selection for rifampicin and chloramphenicol. After growth in this medium colonies were selected on medium containing 10% sucrose in the absence of chloramphenicol. Sucrose induces the *sac*BR gene which encodes levansucrase that converts sucrose to levan. This compound is toxic to many Gram negative organisms. In this way clones still carrying the suicide plasmid were killed leaving exconjugants that had lost the plasmid.

Results. A colony was selected that was chloramphenicol and kanamycin sensitive. PCR amplification of the *thy*A region with the primers ThyA-10 and thyA-11

confirmed that the *thy*AKan fragment (2.6 kb) on the chromosome had been replaced with the Δ*thy*AΔKan fragment (2.1 kb).

Growth of the resulting strain was only observed if the growth medium was supplemented with thymine (200 µg/ml). This strain was named *V.cholerae* JS1569 Δ*thy*AΔKan.

Direct deletion of the *thy*A gene in *V. cholerae*.

For this approach the 5' and 3' sequences flanking the *thy*A gene were used. A novel suicide vector was constructed, pMT SUICIDE-1 (Fig 14) that contains the R6K origin of replication, the *mob* genes from RP4, a chloramphenicol resistance gene and a multicloning site from Litmus 28 (New England Biolabs). Effectively, a modified fragment was constructed in which the *thy*A coding region was replaced by a multicloning site (derived from Litmus 28) leaving only the 5' and 3' region of the *thy*A locus from *V.cholerae*. The resulting plasmid was used to generate a *V. cholerae* strain in which the entire *thy*A gene had been deleted.

As starting material for this construction the pMT SUICIDE-1 plasmid was used (M. Lebens, unpublished).

From the 5' and 3' regions of the *thy*A locus the following PCR primers were designed:

ThyA-33: $^{5'}$GG**A CTA GT**G GGT TTC CTT TTT GCT AT$^{3'}$

corresponding to bases 109 to 126 in the SEQ ID NO:2 (figure 2) (5' region of the *thy*A region) with a *Spe*I site (indicated in bold) and

ThyA-34: $^{5'}$CCC CG**C TCG AG**A CCC TAT TTT GCT GCT AC$^{3'}$
corresponding to the complementary sequence of base 815 to 832 in the SEQ ID NO:2 with a *Xho*I site (indicated in bold) attached to it.

This primer pair gives a PCR fragment of 743 bases corresponding to the 5' flanking region of the *thy*A gene.

ThyA-31: $^{5'}$CGG **GGT ACC** TGG CTT GAT GGG TTT TAT$^{3'}$

corresponding to bases 22 to 39 in the SEQ ID NO:3 (figure 3) (3' region of the *thy*A region) with a *Kpn*I site (indicated in bold) and

ThyA-32: $^{5'}$GA**A GGC CTT** CGC CTC TGC TTG CGA CT$^{3'}$

corresponding to the complementary sequence of bases 731 to 749 in the SEQ ID NO:3 with a *Stu*I site (indicated in bold).

This primer pair gives a PCR fragment of 746 bases corresponding to the 3' flanking region of the *thy*A gene.

As template for the PCR reactions a chromosomal DNA preparation from *V. cholerae* JS1569 was used (Figure 13).

The amplified DNA were digested with the appropriate restriction enzymes and cloned into the pMT-SUICIDE 1 vector (Figure 14 and 15) yielding the plasmid pMTΔ*thy*A *V.cholerae* that contains approximately 700 base-pairs of the 5' region upstreams of the *thy*A gene and the same number of base-pairs of the 3' region downstreams of the *thy*A gene.

This plasmid was transferred to *E.coli* S17-1 and used in conjugation experiments as described above. As recipient the *V. cholerae* JS1569 4.4 strain was used. Matings were done on LB agar supplemented with rifampicin, chloramphenicol and thymine. Exconjugants that had lost the suicide plasmid from the chromosome were selected on the basis of chloramphenicol sensitivity.

Results. A chloramphenicol sensitive and rifampicin resistant colony was selected. PCR amplification with the primers ThyA-10 and ThyA-11 of the *thy*A region resulted in a 1.4 kb fragment from the native *thy*A gene and a 0.6 kb fragment from the Δ*thy*A gene.This confirmed that the *thy*A structural gene on the chromosome had been deleted. Furthermore the bacteria could only grow in medium complemented with thymine. This strain is namned *V. cholerae* JS1569 Δ*thy*A.

Expression of the B subunit of heat-labile enterotoxin from *E. coli* (LTB) and the sj26 glutathione-S-transferase (GST) from *Schistosoma japonicum* in *V. cholerae JS1569 ΔthyA*..

Two expression vectors were constructed, each consisted of the *thy*A gene from *E. coli*, the origin of replication of the high copy-number vector pUC19, the *tac* promotor and the rho-independent *trp*A transcription terminator. In one of the two vectors the *lacI*^{q} gene had been inserted in order to regulate expression from the *tac* promotor which also contained the *lac* operator sequence (figure 16 and 17).

Expression of the LTB protein in *V.cholerae* JS1569 Δ*thy*A strain.

The expression vector shown in figure 16 was electroporated into *V.cholerae* JS1569 Δ*thy*A. Transformants were selected for on MS -agar. Individual colonies were grown up to produce mini-plasmid preps that were checked by restriction enzyme analysis. For expression a transformant was grown in MS medium at 37°C in a shaker culture. The culture medium was harvested and assayed for LTB by the GM1- ELISA.

Results. The culture was found to produce approximately 300 µg/ml of LTB as assayed by the GM1 ELISA. SDS-PAGE and Western blot using an LTB specific monoclonal antibody further verified that the secreted protein was LTB.

Expression of the GST protein in *V.cholerae* JS1569 Δ*thy*A strain

The sj26 glutathione-S-transferase (GST) from *Schistosoma japonicum* was cloned in the expression vector shown in figure 17. This vector is identical to the first except for the sequence of the *lacI*^{q} gene. The *lacI*^{q} allows for controlled expression of recombinant proteins. The vector was electroporated into *V.cholerae* JS1569 Δ*thy*A . Transformants were selected for on MS -agar. Indivudual colonies were grown up to produce mini-plasmid preps that were checked by restriction enzyme analysis. For expression a transformant was grown in MS medium at 37°C in a shaker culture with addition of IPTG.

Results. The recombinant protein was found in the cytoplasm of the *V. cholerae* bacteria. SDS-PAGE and Western blot with a GST specific monoclonal antibody (Pharmacia BioTech, Uppsala) confirmed that GST was expressed. The level of GST expression was more difficult to determine than for LTB since the protein was expressed intracellulary but was judged to be in the same range as for LTB.

References

1. Molin, S., K. A. Gerdes. 1984. Stabilized plasmids. US Patent 4,760,022.

2. Morona, R., and S. R. Attridge. 1987. Non-antibiotic marker system. EPC-A- 0251579.

3. Green, J. M., B. P. Nichols, and R. G. Matthews. 1996. Folate biosynthesis, reduction and polyglutamylation. *In*: F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter and H. E. Umbarger (Eds.) *Escherichia coli* and *Salmonella* cellular and molecular biology. ASM Press Washington D.C. pp665-673.

4. Neill, R. J., B. E. Ivins, and R. K. Holmes. 1983. Synthesis and secretion of the plasmid-coded heat-labile enterotoxin of *Escherichia coli* in *Vibrio cholerae*. Science. **221**: 289-290.

5. Sandkvist, M., M. Bagdasarian, S. P. Howard, and V. J. DiRita. 1995. Interaction between the autokinase EpsE and EpsL in the cytoplasmic membrane is required for extracellular secretion in *Vibrio cholerae*. EMBO J. **14**:1664-1673.

6. Neuhard, J. and R. A. Kelln. 1996. Biosynthesis and conversions of pyrimidines. *In*: F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter and H. E. Umbarger (Eds.) *Escherichia coli* and *Salmonella* cellular and molecular biology. ASM Press Washington D.C. pp580-599.

7. Kaper, J. B., H. Lockman, M. M. Baldini, and M. M. Levine. 1984. A recombinant live oral cholera vaccine. Biotechnology **2**:345-349.

8. Sanchez, J., and J. Holmgren. 1989. Recombinant system for overexpression of cholera toxin B subunit in *Vibrio cholerae* as a basis for vaccine development. Proc. Natl. Acad. Sci. USA. **86**:481-485.

9. Wilson, K. 1994. Preparation of genomic DNA from Bacteria. *In* Current protocols in Molecular Biology (F.A. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J. G. Seidman, J.A. Smith, and K. Struhl, eds.) pp. 2.4.1-2.4.2 John Wiley & Sons, New York.

10. Sheen, J. 1994. High-efficency transformation by electroporation. *In* Current protocols in Molecular Biology (F.A. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J. G. Seidman, J.A. Smith, and K. Struhl, eds.) pp. 1.8.4-1.8.5. John Wiley & Sons, New York.

11. Lebens, M., S. Johansson., J. Osek., M. Lindblad and J. Holmgren. 1993. Large-scale production of *Vibrio cholerae* toxin B subunits for use in oral vaccines. Biotechnology. 11:1574-1578.

12. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.

13. Program Manual for the Wisconsin Package. Version 8. September 1994. Genetics Computer Group, 575 Science Drive, Madison Wisconsin.

14. Milton, D. L., A. Nordqvist, and H. Wolf-Watz. 1992. Cloning a metalloprotease gene involved in the virulence mechanism of *Vibrio anguillarum* J. Bacteriol. 174:7235-7244.

15. Milton, D. L., R. O'Toole, P. Högstedt, and H. Wolf-Watz. 1996. Flagellin A is essential for the virulence of *Vibrio anguillarum* J. Bacteriol. 176:1310-1319.

16. Belfort, M., G. Maley, J. Pedersen-Lane and F. Maley. 1983. Primary tructure of the *Escherichia coli thy*A gene and its thymidylate synthase product. Proc. Natl. Acad. Sci. USA **80**: 4914-4918.

17. Fleischmann, R.. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult,C.J., Tomb, J.-F., Dougherty, B. A., Merrick, J. M., McKenney, K., Sutton, G., FitzHugh, W., Fileds, C. A., Gocayne, J. D., Scott, J. D., Shirely, R.., Liu, L.-I., Glodek, A., Kelley, J.M. Weidman, J. F., Phillips, C. A., Spriggs, T., Hedblom, E., Cotton, M. D., Utterback, T. R., Hanna, M. C., Nguyen, D. T., Saudek, D. M., Brandon, R.. C., Fine, L. D., Fritchman, J. L., Fuhrmann, J. L., Geoghagen N.S.M., Gnehm, C. L., McDonald, L. A., Small, K. V., Fraser, C. M., Smith, H. O., and J. C. Venter. 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* RD. Science **269**:496-512.